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Research Article

Binding of Vitamin K₁(Phylloquinone) to Human Serum Albumin(HSA):Spectroscopic studies.

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Abstract: The interaction of hydrophobic vitamin (vitamin K₁) with human serum albumin (HSA) at physiological (pH 6.9- 7.4) has been studied using UV-VIS spectrometer, and an FT-IR spectroscopy. The interaction of hydrophobic vitamin (vitamin K₁) with HSA has been investigated by using UV-absorption, and Fourier transforms infrared (FT-IR) spectroscopy. The binding constant of vitamin K₁ has been determined by UV-absorption. The value of the binding constant for vitamin K₁ -HSA is calculated at room temperature 293 K and it was determined as 60 M⁻¹. FT-IR spectroscopy with Fourier self- deconvolution technique and second derivative resolution enhancement procedures were applied in the analysis of the amide I, amid II, and amid III regions to determine the protein secondary structure and hydrophobic vitamin binding mechanisms. All peaks positions in the three amide regions (amid I, amide II and amide III) have been assigned and any changes due to concentration changes have been investigated. The FTIR spectra measurements indicate a change in the intensity of absorption bands due to change in the concentrations in drugs. In addition a larger intensity decrease in the absorption band of α -helix relative to that of β -sheets has been observed. This variation in intensity is related indirectly to the formation of H-bonding in the complex molecules, which accounts for the different intrinsic propensities of α -helix and β -sheets.

Keywords: Vitamin K₁; amide I-III ; binding mode; binding constant; protein secondary structure; Fourier transform IR; UV-spectroscopy, Fluorescence spectroscopy.

INTRODUCTION

Vitamins are organic, low molecular weight components of the diet required by the organisms in very small amounts to perform specific cellular functions. They are not synthesized by organism, so it must be ingested with the diet or acquired in some other way¹⁻³.

Hydrophilic- vitamins are nine vitamins which are soluble in water. Where Hydrophobic- vitamins are four vitamins soluble in fat, and they are released, absorbed, and transported with the fat of the diet². Vitamin K is a Fat- soluble vitamin which was named from the word Koagulation which is the German spelling for coagulation⁴. Vitamin K functions as a coenzyme and is involved in the synthesis of a number of proteins involved in blood clotting and bone metabolism^{5, 6}. Compounds with vitamin K₁ activity all have a 2-methyl 1, 4-naphthoquinone ring and a side chain at the 3-position as shown in **Fig. (1)**. In nature, this 3-substituent has an isoprenoid structure with varying lengths and degrees of saturation depending on the organism by which it is synthesized.

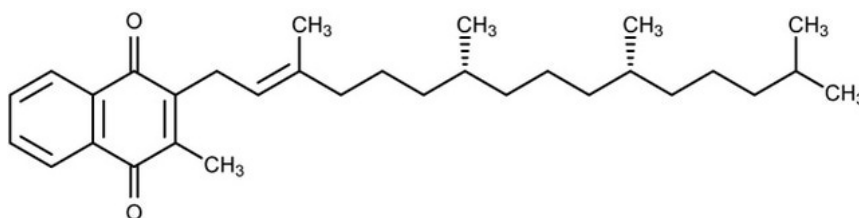


Figure 1: Chemical structure of Vitamin K₁.

Phylloquinone and menaquinones are natural forms of vitamin K₁ that are important cofactors in blood-clotting factors⁷. Phylloquinone is the predominant⁸ form of vitamin K and is provided mainly by plant foods, especially leafy green vegetables, and certain legumes⁹.

Recently, it was demonstrated a correlation between vitamin K₁ and sphingolipids concentrations in rat brain¹⁰; moreover this vitamin present a protective effect on aging retina, the sparing effect being most evident in the inner plexiform layer and in the photoreceptor inner and outer segments¹¹ ssays with vitamin K remain to be made on human.

All vitamins are now produced commercially. The commercial production of vitamins is primarily by chemical synthesis. Fat soluble vitamins are also commercially isolated from natural sources¹². Human serum albumin (HSA) is the most abundant protein in blood plasma, which has a number of physiological functions involving transport of various endogenous and exogenous chemicals, e.g., pharmaceuticals. The distribution, metabolism, and toxicity of such chemicals are significantly affected by their binding to HSA. Moreover, there is evidence of secondary structural change of HSA induced by its interaction with exogenous chemicals which will affect the physiological function¹³ of HSA. Therefore, the interaction between HSA and pharmaceuticals are of imperative and importance. Various techniques have been used to study these interactions, e.g., fluorescence spectrometry^{14,15}, FT-IR, Raman spectrometry¹⁶, circular dichroism(CD)¹⁷, equilibrium dialysis¹⁸, isothermal titration calorimetry¹⁹, and capillary electrophoresis²⁰. Compared with other analytical techniques, fluorescence spectrometry is a conventional and powerful method to study the molecular interactions involving proteins, owing to its sensitivity, rapidity and simpleness. From analysis of the HSA spectrum before and after the addition of ligands, a great amount of information could be obtained, such as the binding constant and mode, the microenvironment information of the fluorophore and so on²¹. In this study, we have investigated the interaction of

Vitamin K₁ with HSA by means of FT-IR, UV/VIS, and fluorescence spectrophotometer. Infrared spectroscopy provides measurements of molecular vibrations due to the specific absorption of infrared radiation by chemical bonds. It is known that the form and frequency of the Amide I band, which is assigned to the C=O stretching vibration within the peptide bonds is very characteristic for the structure of the studied protein. From the band secondary structure, components peaks (α -helix, β -strand) can be derived and the analysis of this single band allows elucidation of conformational changes with high sensitivity²². This work will be limited to the mid-range infrared, which covers the frequency range from 4000 to 400 cm⁻¹. This wavelength region includes bands that arise from three conformational sensitive vibrations within the peptide backbone (Amides I, II and III) of these vibrations, Amide I is the most widely used and can provide information on secondary structure composition and structural stability²³⁻²⁵. One of the advantages of infrared spectroscopy is that it can be used with proteins that are either in solution or in thin film. In addition there is a growing body of literature on the use of infrared to follow reaction kinetics and ligand binding in proteins, as well as a number of infrared studies on protein dynamics.

MATERIALS AND METHODS

Materials: HSA (fatty acid free), vitamin K₁ (Phylloquinone) were purchased from Sigma Aldrich chemical company and used without further purifications. The data were collected using samples in the form of thin films for FT-IR measurements and liquid form for UV-VIS.

Preparation of stock solutions: Preparations of the thin film samples required three stock solutions as described below:

HSA was dissolved in 25% ethanol in phosphate buffer Saline and at physiological (pH 6.9- 7.4), to a concentration of (80mg/ml), and used at final concentration of (40 mg/ml) in the final vitamin- HSA solution.

Vitamin K₁ with molecular weight of (450.7 g.mol⁻¹) and density (0.984 g/ml), was dissolved in 25% ethanol in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for two days to ensure that all the amount of vitamin E was completely dissolved.

HSA concentration was fixed at 40 mg.ml⁻¹ in all samples. However, the concentration of vitamin K₁ in the final HSA-vitamin K₁ solutions was decreased such that the molecular ratios (HSA: vitamin K₁) are 1:20, 1:10, 1:5, 1:2, and 1:1. All samples were made by mixing equal volume from HSA to equal volume from different concentration of vitamin K₁.

Silicon windows (NICODOM Ltd) were used as spectroscopic cell windows. The optical transmission is high with little or no distortion of the transmitted signal. The 100% line of a NICODOM silicon window shows that the silicon bands in the mid- IR region do not exhibit total absorption and can be easily subtracted. 40 μ L of each sample of HSA –vitamin K₁ was spread on a silicon window and an incubator was used to evaporate the solvent, to obtain a transparent thin film on the silicon window. All solutions were prepared at the same time for one run at room temperature 25^oC.

UV-absorption spectroscopy: The absorption spectrum of a measurements were performed by a Nano-Drop ND-1000 Fluor spectrometer at 25^oC. The excitation had been done at the wavelength of 210 nm and the maximum emission wavelength is at 280 nm. The excitation source comes from one of three solid-state light emitting diodes (LED's). A 2048-element CCD array detector covering 220–750 nm,

is connected by an optical fiber to the optical measurement surface. The UV absorbance intensity of HSA increased with the increasing of vitamin K₁ concentration as shown in **Fig.(2)**. In addition, the binding of the vitamin K₁ to HSA resulted in a slight shift of the HSA absorption spectrum. These results clearly indicated that an interaction and some complex formation occurred between HSA and vitamin K₁, and also indicated that the peptide strands of protein molecules extended more upon the addition²⁶ of vitamin K₁ to HSA. It is evident from the spectra of the pure vitamins the little or no absorption effect which supports that the resulted peaks are due to the interaction between the vitamin K₁ and HSA.

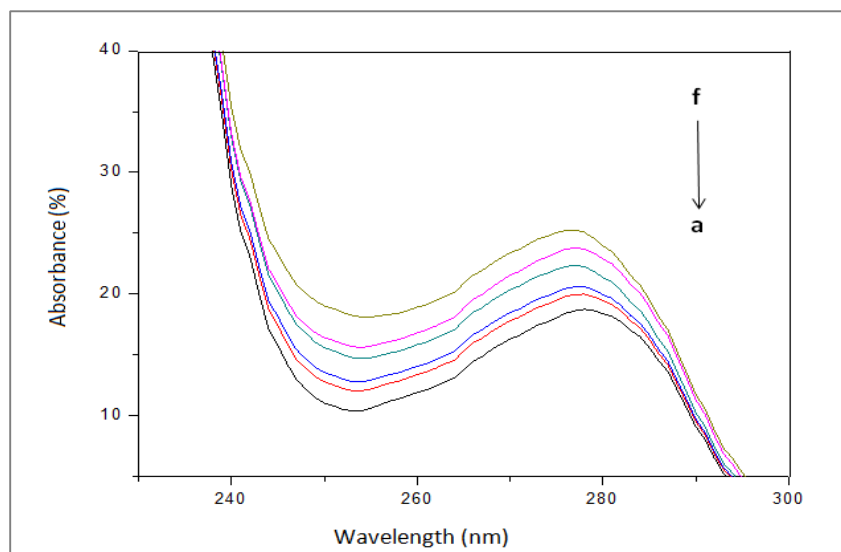


Figure 2: UV-VIS absorbance spectra of HSA with different concentrations of Vitamin K₁ (a=1:0, b=1:1, c=1:2, d=1:5, e=1:10, f=1:20)

The emission spectra were recorded for free HSA (40 mg/mL) and for its complexes with vitamin K₁ solutions with different concentrations of HSA: Vitamin K₁. The solution of vitamins and HSA were incubated for 1 h (at 20°C) before spectroscopic measurements were taken.

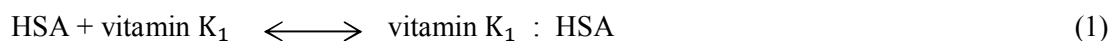
FT-IR Spectroscopy Experimental Procedures; The FT-IR measurements were obtained on a Bruker IFS 66/S spectrophotometer equipped with a liquid nitrogen-cooled MCT detector and a KBr beam splitter. The spectrometer was continuously purged with dry air during the measurements. The absorption spectra were obtained in the wave number range of 400–4000 cm⁻¹. A spectrum was taken as an average of 60 scans to increase the signal to noise ratio, and the spectral resolution was at 4 cm⁻¹. The aperture used in this study was 8 mm, since we found that this aperture gives best signal to noise ratio. Baseline correction, normalization and peak areas calculations were performed for all the spectra by OPUS software. The peak positions were determined using the second derivative of the spectra. The infrared spectra of HSA, vitamin E–HSA complexes, and vitamin D –HSA mixtures, were obtained in the region of 1000–1800 cm⁻¹. The FT-IR spectrum of free HSA was acquired by subtracting the absorption spectrum of the buffer solution from the spectrum of the protein solution. For the net interaction effect, the difference spectra {(protein and vitamin K₁ solution) – (protein solution)} were generated using the featureless region of the protein solution 1800–2200 cm⁻¹ as an internal standard²⁷.

The accuracy of this subtraction method is tested using several control samples with the same protein or drug concentrations, which resulted into a flat base line formation. The obtained spectral differences were used here, to investigate the nature of the drug–HSA interaction. We had also used

ELAB 12/05 thermo system to directly and simultaneously determine the thermo-dependent structural changes of drug-protein complexes.

RESULTS AND DISCUSSION

Analysis of UV-absorption spectroscopy of HSA by Vitamin K₁: The value of the binding constant K between HSA and vitamin K₁ can be determined using the data resulted from the UV-VIS spectroscopy according to the method described earlier in many published articles^{26,28,29}. By assuming that there is only one type of interaction between HSA and vitamin K₁ in aqueous solution, which leads to establish equations (1) and (2) as follows:



$$K = \frac{[\text{vitamin K}_1 : \text{HSA}]}{[\text{vitamin K}_1][\text{HSA}]} \quad (2)$$

The value of the binding constant K can be calculated using the following equation²²:

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K(A_\infty - A_0)} \times \frac{1}{L} \quad (3)$$

Where A₀ the initial absorption of the free protein at 280 nm is, A_∞ is the final absorption of the ligated protein, and A is the recorded absorption at different vitamin K₁ concentrations (L)²⁸.

The double reciprocal plot of 1/(A – A₀) versus 1/L is linear as in **Fig.(3)** and the binding constant (K) can be estimated from the ratio of the intercept to the slope which equals 60 M⁻¹. The value of the binding constant calculated show a weak HSA - vitamin K₁ interaction with respect to the other strong ligand–protein complexes with binding³⁰ constants ranging from 10⁵ to 10⁶ M⁻¹. The reason for the low stability of the vitamin K₁ :HSA complexes can be attributed to the presence of mainly hydrogen bonding interaction between protein donor atoms and the vitamin K₁ polar groups or an indirect vitamin K₁-protein interaction through water molecules³¹.

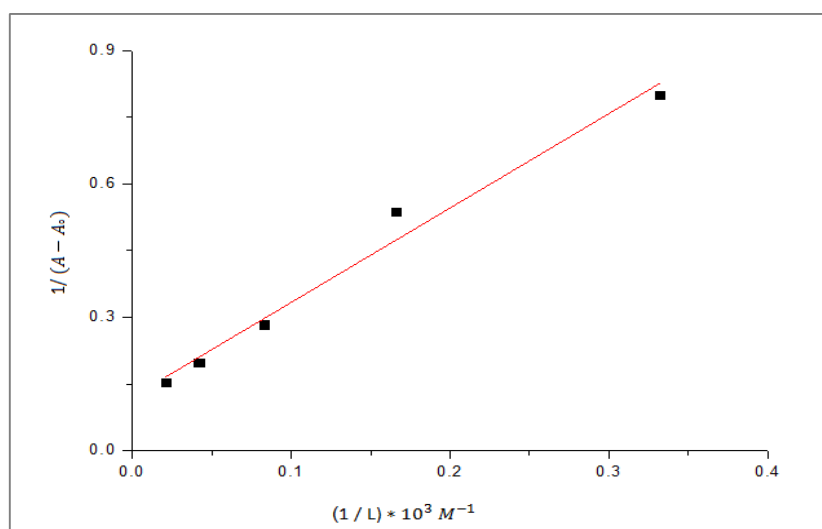


Figure 3: The plot of 1/(A – A₀) vs. 1/L for HSA with different concentrations of VitaminK₁

FT-IR Spectroscopy: Mid- IR spectroscopy is one of the earliest techniques for studying the protein secondary structure³²⁻³⁴. FTIR spectroscopy can be used to determine the protein secondary structure in a wide range of environments (e.g., in a solution as in the solid state) requiring less time and sample than other spectroscopic techniques^{33, 35}. FTIR spectroscopy provides also information about the interaction between the protein and the ligand³⁶.

FTIR spectrum of a protein is composed of many vibrational bands arising from different functional groups such as N-H, C = O, and the amide groups³⁵. So, IR spectra of proteins contain a number of the amide bands, the most popular ones in the ranges ($1700\text{-}1600\text{ cm}^{-1}$), ($1600\text{-}1480\text{ cm}^{-1}$), ($1330\text{-}1220\text{ cm}^{-1}$) are related to the bands which are called (amides I, II and III) respectively^{28, 37}. Amide I band is primarily due to C = O stretching vibrations of the amide groups³⁴. This band is the most widely used to study the protein; because is more sensitive to the change in their secondary structure than other amide bands^{36, 37}. Amide II band is primarily due to the N-H bending vibrations combined with C-N stretching vibration³⁸. Where amide III region is due to the C-N stretching vibrations coupled with in plane N-H bending vibrations²⁶, which are the same to that of amide II, albeit with a different sign in the combination of the coordinate³⁹.

Two major absorbance bands appeared in the second derivative spectra for free HSA as shown in **Fig. (4.A)**. These bands which are at peak positions 1656 cm^{-1} and 1546 cm^{-1} are related to the amide I and amide II bands respectively.

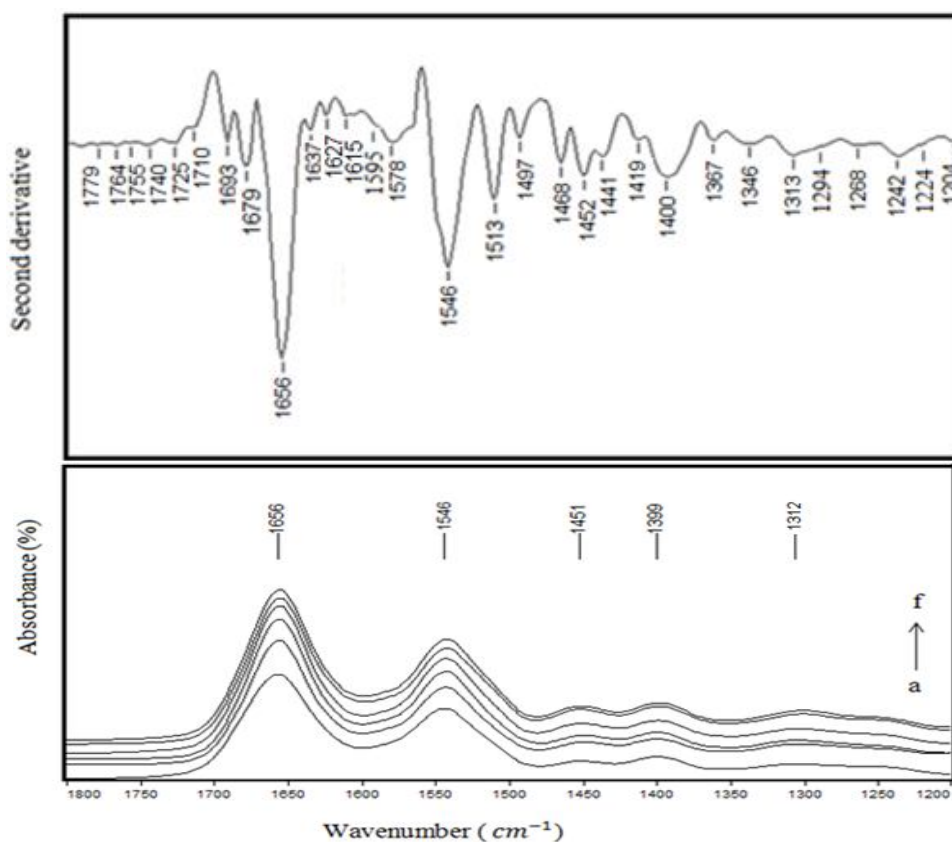


Figure 4: The spectra of (A): HSA free (second derivative) and (B): (a, b, c, d, e) HSA – VitaminK₁ with molecular ratios (1:0, 1:2, 1:5, 1:10, 1:20), respectively.

The absorbance spectra for HSA- Vitamin K₁ complexes with different molecular ratios of VitaminK₁ are shown in **Fig.(4.B)**, it is clear that the absorbance intensity of HSA increases as the VitaminK₁ ratios increases; this increase in the intensity is due to the interaction between HSA and VitaminK₁.

The peak positions for HSA- vitamin K₁ complexes absorption bands with different molecular ratios of vitamin K₁ are listed in **(Table 1)**.

Table -1: Band assignments in the absorbance spectra of HSA with different VitaminK₁ molecular ratios for amide I, amide II, and amide III regions.

Bands	HAS Free (%)	HAS- V.K ₁ 1:1 (%)	HAS- V.K ₁ 1:2 (%)	HAS- V.K ₁ 1:5 (%)	HAS- V.K ₁ 1:10 (%)	HAS- V.K ₁ 1:20 (%)
Amide I (1600-1700)	1615	1614	1615	1614	1614	1613
	1627	1627	1628	1628	1629	1629
	1637	1637	1637	1637	1638	1638
	1656	1656	1657	1656	1657	1658
	1679	1679	1680	1680	1681	1682
	1693	1692	1693	1693	1694	1694
Amide II (1480-1600)	1497	1497	1497	1497	1496	1496
	1513	1513	1512	1513	1513	1514
	1546	1546	1546	1547	1547	1547
	1578	1578	1579	1578	1579	1579
	1595	1595	1594	1594	1593	1593
Amide III (1220-1330)	1224	1224	1224	1224	1224	1223
	1242	1242	1441	1441	1242	1241
	1268	1268	1267	1268	1269	1269
	1294	1294	1294	1293	1293	1293
	1313	1313	1313	1312	1314	1314

For amide I band, the peak positions for HSA are shifted after mixing with vitamin K₁ as follows: 1615 to 1613cm⁻¹, 1627 to 1629cm⁻¹, 1637 to 1638 cm⁻¹, 1656 to 1658cm⁻¹, 1679 to 1682cm⁻¹, and 1693 to 1694cm⁻¹. For amide II region, the peak positions have been shifted as follows: 1497 to 1496 cm⁻¹, 1513 to 1514 cm⁻¹, 1546 to 1547 cm⁻¹, 1578 to 1579 cm⁻¹, and 1595to 1593cm⁻¹. In amide III region, the peak positions have also been shifted as follows: 1224 to 1223cm⁻¹ , 1242 to 1241 cm⁻¹, 1268 to 1269 cm⁻¹, 1294 to 1293cm⁻¹, and 1313 to 1314 cm⁻¹.

The difference spectra between (HSA +vitaminK₁) and HSA as shown in (Fig. 5) is performed to obtain the intensity variations resulted due to the interaction between HSA and vitamin K₁ .

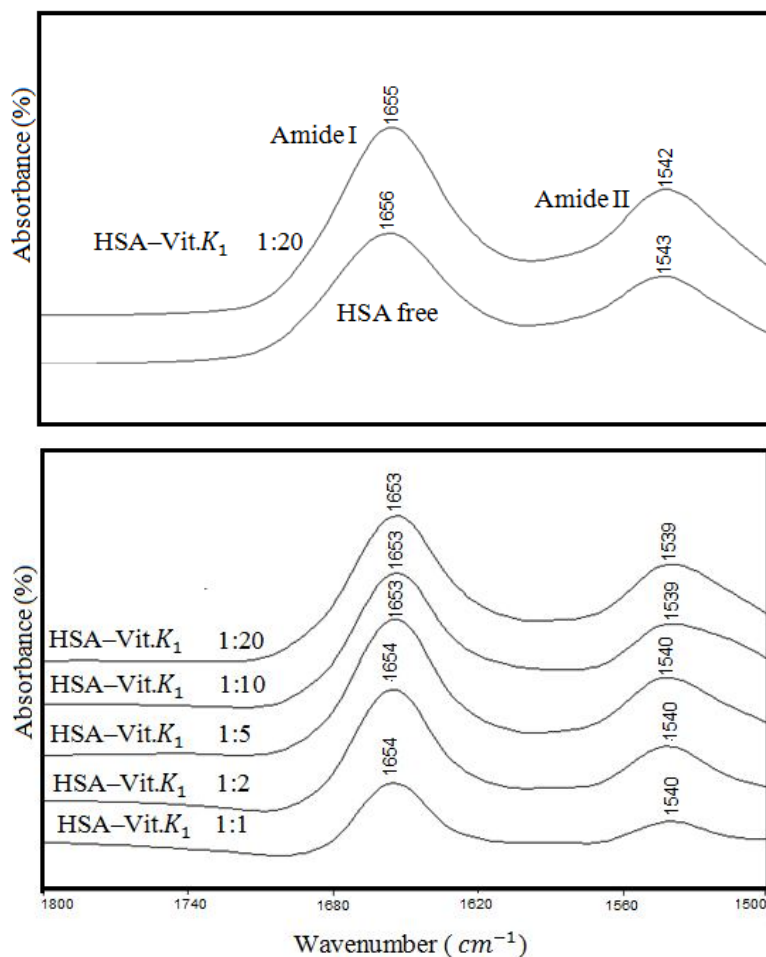


Figure 5: FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different VitaminK₁ molecular ratios in the region 1800 – 1500 cm^{-1}

In amide I region, a strong positive peak at 1654 cm^{-1} is resulted at the lowest molecular ratio of vitamin K₁ and at 1653 cm^{-1} at the highest molecular ratio of it. In amide II region, a weaker positive peak at 1540 cm^{-1} is resulted at the lowest molecular ratio of vitaminK₁ and at 1539 cm^{-1} at the highest molecular ratio of it.

By comparison between the absorption spectra for free HSA which are the top two curves in **Fig.(4)** and the difference spectra of HSA and its complexes with different vitaminK₁ molecular ratios, it is clearly appeared that the positive feature became stronger as vitaminK₁ molecular ratio was increased with a little shift in their positions. This is related to the increase in the intensity of FTIR absorption spectra of HSA - vitaminK₁ complexes as vitaminK₁ molecular ratio was increased which is due to the interaction between HSA and vitamin K₁ by forming hydrogen bonds with C = O and C-N groups of the protein, and

to increase of the protein α -helix structure which is also related to the interaction^{26, 31, 40} between HSA and vitaminK₁.

The observed amide band contour of the protein consists of overlapping component bands representing different secondary structures such as α - helices, β - sheets, turns and random structures which exhibit characteristic frequencies and intensities due to the differences in the hydrogen bonds in these structures^{33, 35}.

In this work, the quantitative determination of the protein secondary structure for free HSA and HSA-vitamin K₁ complexes in dehydrated films was performed according to the procedure described by Byler and Susi³⁴.

Various data- processing techniques such as Fourier Self Deconvolution (FSD), curve fitting, and second derivative have been proposed to enhance the resolution of the spectrum for the overlapping amide bands of proteins allowing the intrinsically broad components to be narrowed to estimate quantitatively the relative contributions of the different types of secondary structures^{33, 34}.

The component bands of amide I, II, and III regions which are showed in (Table. 2) were assigned according to the frequency of its maximum a raised after baseline corrections and FSD have been applied to the absorbance spectrum. For amide I band which is in the range (1700- 1600 cm^{-1}) the ranges for the bands of the secondary structure components were assigned as follows: (1609-1629 cm^{-1}) for β - sheets, (1629 -1647 cm^{-1}) for random coils, (1647-1672 cm^{-1}) for α - helices, (1674-1688 cm^{-1}) for turn structure, and (1688-1700 cm^{-1}) for β - antiparallel. For amide II band which is in the range (1600-1480 cm^{-1}) the ranges for the band components were assigned as follows: (1489-1505 cm^{-1}) for β - sheets, (1505- 1523 cm^{-1}) for random coils, (1528-1562 cm^{-1}) for α - helices, (1562-1588 cm^{-1}) for turn structure, and (1588-1600 cm^{-1}) for β - antiparallel. For amide III which is in the range (1220- 1330 cm^{-1}), the ranges for the band components were assigned as follows: (1220-1251 cm^{-1}) for β - sheets, (1251-1288 cm^{-1}) for random coils, (1288-1297 cm^{-1}) for turn structure, (1300-1330 cm^{-1}) for α - helices.

Specific areas of the protein secondary structures are needed to be calculated for quantization of the respective absorbance, or for a comparative analysis. For a deconvolved spectrum, the peak area assessment signifies a summation of all the absorbance values over the wave number range in which the band show up by means of integration⁴², then the percentages of each secondary structure were calculated by dividing the integrate area for each component by the total area of the amide band. The percentages of each secondary structure for free HSA and for HSA -vitamin K₁ complexes in amides I, II, and III are listed in (Table.2).

The second derivative and curve fitting was performed on the absorption spectra for HSA free and the HSA- vitaminK₁ of highest molecular ratio as shown in **Fig. (6)**. It is clearly observed that the percentages of α - helices increases, whereas the percentages of β - sheets decreases as the molar ratios of vitaminK₁increase in all the amide regions (I, II and III); the increase of α - helices intensity percentage in favor of the reduction of β - sheets which indicates that vitaminK₁interacts with HSA is related to the folding of the protein in the presence of vitaminK₁ as a result of the formation of H- bonding with the C = O bond assuming partial double bond character due to a flow of electrons from C – N to C = O bond which increases the intensity of the α - helices

Table-2: Secondary structure determination for amide I, amide II, and amide III regions in HSA and its VitaminK₁ complexes.

<i>2nd structure</i>	HSA Free (%)	HSA –V.K ₁ 1:1 (%)	HSA –V.K ₁ 1:2 (%)	HSA –V.K ₁ 1:5 (%)	HSA –V.K ₁ 1:10 (%)	HSA –V.K ₁ 1:20 (%)
			Amide I			
β -sheets (cm^{-1}) (1609-1623) (1688-1700)	20	19	18	16	16	15
Random (cm^{-1}) (1629-1647)	10	11	12	12	13	13
α -helix (cm^{-1}) (1647-1672)	56	57	58	58	58	59
Turn (cm^{-1}) (1674-1688)	14	13	12	12	11	11
			Amide II			
β -sheets (cm^{-1}) (1489-1505) (1588-1600)	22	22	21	20	20	19
Random (cm^{-1}) (1505- 1523)	10	10	10	11	12	13
α -helix (cm^{-1}) (1528-1562)	53	53	54	55	55	56
Turn (cm^{-1}) (1562-1588)	15	15	15	14	13	12
			Amide III			
β -sheets (cm^{-1}) (1220-1251)	20	20	19	19	19	18
Random (cm^{-1}) (1251-1288)	16	16	16	17	17	17
Turn (cm^{-1}) (1288-1297)	11	11	11	10	10	10
α -helix (cm^{-1}) (1300-1330)	50	50	51	51	51	52

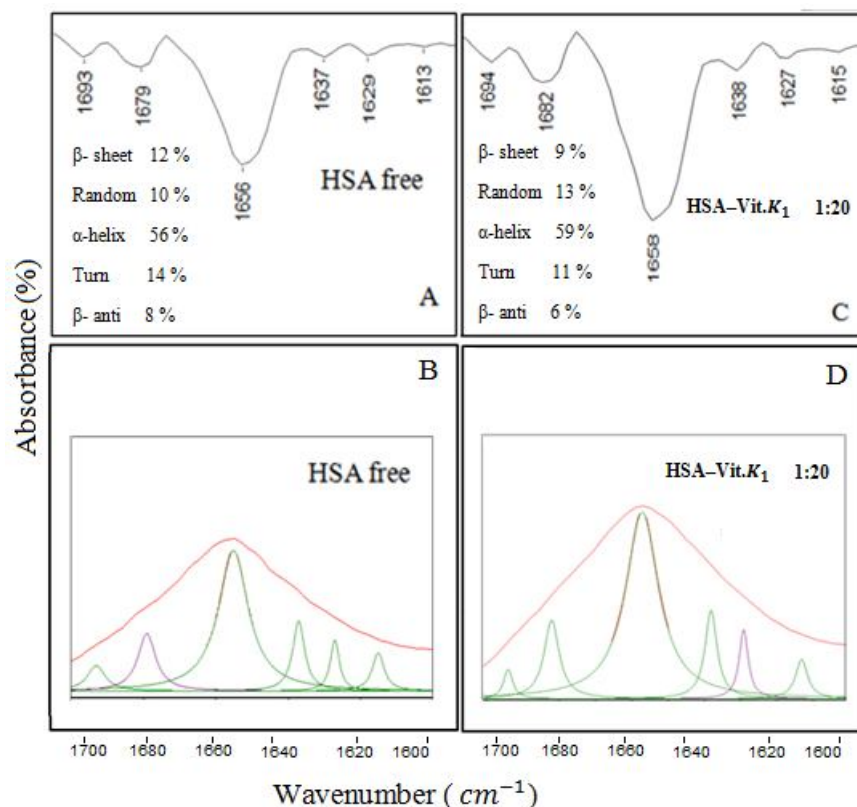


Figure 6: Second derivative resolution and curve fitted amide I region (cm^{-1}) and secondary structure determination of the free HSA (A, B) and its Vitamin K₁ complexes (C, D) with 1:20 protein: vitamin ratio.

CONCLUSION

The interaction of HSA with vitamin K₁ is almost similar to the interaction of it with the vitamin A components (retinol and retinoic acid), whereas, it is almost different from the interaction between it with vitamin D and vitamin E.

The interaction between HSA and vitamin K₁ was investigated by means of UV-VIS spectrophotometer, and FT-IR spectroscopy. Using UV-Vis absorption spectrophotometry, it was found that the value of the binding constant between HSA and vitamin K₁ equals 60 M^{-1} , which indicates a weak binding between them in comparison with the other hydrophobic vitamins which have a binding constants with value equals $1.32 \times 10^5 \text{ M}^{-1}$, $3.33 \times 10^5 \text{ M}^{-1}$, $1.21 \times 10^2 \text{ M}^{-1}$, $6.8 \times 10^1 \text{ M}^{-1}$ for retinol, retinoic acid, vitamin E and vitamin D respectively [40].

FTIR spectroscopy results indicate that the absorbance intensity increases as the molecular ratios of vitamin K₁ increase. This increasing in intensity is related to the increase of the absorbance intensity for α -helices which indicates that the interaction between HSA and vitamin K₁ leads to the folding of the protein by forming new H-bonding in the C = O bond, assuming a partial double bond character due to a flow of electrons from C – N to C = O bond which is the reason of increasing the intensity of the α -helices. This increasing of this intensity is similar to the increasing that was observed of the interaction of vitamin A

components with HSA (retinol and retinoic acid) with HSA have been studied, but with different absorbance ratios⁴⁰.

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